ORIGINAL ARTICLE

Determination of in vitro postantibiotic effects in *Staphylococcus aureus* and *Escherichia coli* by [^{3}H]thymidine incorporation

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Postantibiotic effects (PAE) and control-related effective regrowth time (CERT) of dicloxacillin, vancomycin, rifampin and gentamicin in *Staphylococcus aureus* and imipenem, gentamicin, tobramycin, doxycycline and rifampin in *Escherichia coli* were measured by standard viability counting and [^{3}H]thymidine incorporation. For PAE determination, the two methods correlated well; $r^2 = 0.821$ for *S. aureus* and $r^2 = 0.939$ for *E. coli*. For viable counts below the detection limits of 10^5 to 10^6 $\log_{10}$ CFU/mL, the PAE was overestimated by the [^{3}H]thymidine method. Quantitation of CERT by both methods showed a good correlation, $r^2 = 0.867$ for *S. aureus* and $r^2 = 0.997$ for *E. coli*. Measuring [^{3}H]thymidine incorporation in bacteria is a novel alternative method for the determination of PAE and CERT.

Key words: Postantibiotic effect, [^{3}H]thymidine incorporation, *S. aureus, E. coli*

INTRODUCTION

The postantibiotic effect (PAE), the period of growth suppression after short exposure to antibiotics, is a feature of several organism–antibiotic combinations [1,2]. The clinical relevance of the PAE pertains to its impact on antimicrobial dosing, where drugs inducing long PAEs may be administered with longer dosing intervals than currently employed without loss of efficacy [2]. This has been demonstrated in clinical medicine with once-daily dosing of aminoglycosides [3–5].

The standard method of PAE determination by viability counting [2] is rather time-consuming and tedious, and various alternative methods have therefore been devised. They include the bioluminescence assay of intracellular ATP [6–9], measurements of optical density [10–12], electrical impedance [13], and conductance [14]. Furthermore, bacterial morphology, as estimated by microscopy [15] or electron microscopy [16] or automatically, employing a Coulter counter [17], has also been used for PAE measurements. We have previously described a novel method where PAE was quantitated by measuring bacterial CO_2 generation [18].

Control-related effective regrowth time (CERT) is a combined pharmacodynamic parameter which incorporates both bacterial kill and PAE. While for some antibiotic–organism combinations the duration of PAE has been shown to be variable depending on the method used, there is some evidence that values for CERT may be less method dependent [19].

The purpose of this study was to evaluate whether [^{3}H]thymidine incorporation could be used for PAE and CERT determination in vitro as compared to the standard method of viability counting.

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MATERIALS AND METHODS

Organisms and media

The organisms used in the study were *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923.

Mueller–Hinton broth (MHB, Difco Laboratories, Detroit, Michigan) served as culture medium.

Antibiotics and chemicals

Rifampin was supplied by Ciba-Geigy (Basel, Switzerland), doxycycline by Delta (Hafnarfjördur, Iceland), gentamicin by Roussel Laboratories Ltd (Uxbridge, UK), imipenem by Merck Sharp & Dohme International (Rahway, New Jersey, USA), dicloxacillin by Bristol Sermoneta (Latina, Italy), and tobramycin and vancomycin by Eli Lilly & Co. (Indianapolis, Indiana, USA). Stock solutions were prepared in sterile saline except imipenem and rifampin, for which phosphate-buffered saline and methanol were used, respectively. Fresh stock solutions were prepared daily for imipenem and rifampin but stored at −20 °C until use for other antimicrobials. Minimum inhibitory concentrations (MICs) were determined by a standard microtiter dilution method [20].

6-[^{3}H]thymidine (specific activity 5.0 Ci/mmol) was purchased from Amersham International (Amersham, UK) and trichloroacetic acid (TCA) from Merck (Merck, Darmstadt, Germany). Toluene with 5 g of 2,2′-*p*-phenylen-bis-(phenyloxazol) (POPOP) and 0.12 g of 2,5-diphenyloxazol (PPO) (Merck) per liter served as scintillation fluid.

Inoculum and organism–antibiotic combinations

Before each experiment, three to four colonies of the test organism were transferred to 5 mL of MHB, diluted serially after 6–8 h of growth and grown overnight at 35.5 °C. Subsequently, the culture was adjusted to an inoculum of ~10^7 CFU/mL in the logarithmic phase of growth. The [^{3}H]thymidine incorporation was studied in each organism after 1 h of exposure to the following antibiotics (concentration in multiples of MIC): *S. aureus* after exposure to dicloxacillin (2×, 4× and 8× MIC), gentamicin (2× MIC), vancomycin (2× and 4× MIC) and rifampin (2× and 4× MIC), and *E. coli* after exposure to gentamicin (0.5×, 1× and 2× MIC), tobramycin (2× MIC), doxycycline (4× and 8× MIC), rifampin (2× and 4× MIC), and imipenem (1× and 2× MIC).

Drug removal

After 1 h of exposure to antibiotics, the bacterial culture was diluted 100-fold in fresh prewarmed MHB in order to remove the antibiotics. This frequently resulted in bacterial counts equal to or less than 10^3 CFU/mL in experiments with very bactericidal drugs. This proved to be below the threshold of detection for [^{3}H]thymidine incorporation, and as a result DNA synthesis was difficult to quantitate in these experiments. In order to get higher bacterial counts in some of the experiments the drug was removed by filtration of the culture through filters with 0.45 μm pore size (Millipore HA, Millipore Corporation, Bedford, MA). The filters were washed with 100 mL of sterile 0.9% NaCl, followed by 10 mL of MHB. Subsequently, the filter was placed in fresh, prewarmed MHB, shaken vigorously for 2 min and removed. The unexposed control organisms were processed in the same manner.

[^{3}H]thymidine incorporation

A modified method of Engle et al. was used [21]. After the drug removal procedure, 1 mL of each of the *E. coli* and *S. aureus* cultures was pulsed at 1.5 h intervals with 10 μCi 6-[^{3}H]thymidine for exactly 10 min.

The bacteria were then lysed with ice-cold TCA, final concentration 10% w/v. The lysed bacteria were transferred to tissue culture microtiter plates, 200 μl in each well at each time point in tri- or quadruplicate.

The suspension was then harvested with a cell harvester (Titertek®, Skatron, Norway) on glass filters and each well washed carefully with 10 mL of sterile water. The glass filters were air-dried for several hours and the radioactivity determined with a 1214 β-liquid scintillation counter (LKB®, Wallac, Turku, Finland).

The counts per minute were used as an indicator of rate of [^{3}H]thymidine incorporation at the time points. Correction was made for background radioactivity. Viable counts were estimated at 1.5-h intervals by serial dilution in ice-cold 0.9% NaCl and plating on MH agar.

Initial studies revealed that [^{3}H]thymidine incorporation was unmeasurable if the number of viable bacteria was <4 $\log_{10}$(CFU/mL). Thus, experiments with lower post-drug removal inocula are not included in the analysis of data for PAE determination.

PAE

The duration of the PAE determined by viable counts (PAE_{vc}) was calculated using the equation PAE = $T - C$, where C is the time required by control organisms to grow 1 $\log_{10}$ CFU/mL, and T is the time necessary for antibiotic-treated bacteria to increase by 1 $\log_{10}$ CFU/mL after drug removal [2]. For [^{3}H]thymidine incorporation, the PAE_{th} was similarly defined as the difference in time for radioactivity of antibiotic-treated and control organisms to increase 1 $\log_{10}$ counts/min.

CERT

The duration of the CERT determined by viable counts was defined as CERT = $T - C$, where C is the time needed for the control to grow 1 $\log_{10}$ CFU/mL after drug removal, and T represents the time taken for the exposed culture to reach the same viable count. For

CERT determined by [^{3}H]thymidine incorporation, the same definition was used, with C being the time needed for the control to increase 1 $\log_{10}$ counts/min after drug removal and T representing the time needed for the exposed culture to reach the same radioactivity.

Analysis of data

The counts per minute values from the triplicates or quadruplicates were averaged. The correlation between the two different methods of PAE measurements was calculated by least-squares linear regression.

RESULTS

For *E. coli* ATCC 25922 the MICs were: gentamicin 1.0 mg/L, tobramycin 2.0 mg/L, imipenem 0.125 mg/L, rifampin 16 mg/L and doxycycline 2.0 mg/L. For *S. aureus* ATCC 25923 the MICs were: gentamicin 0.125 mg/L, dicloxacillin 0.25 mg/L, vancomycin 1.0 mg/L, and rifampin 0.015 mg/L.

An example of a typical experiment involving growth curves and counts/min for *E. coli* after 1 h of exposure to doxycycline, 4×MIC, is presented in Figure 1.

Comparison of duration of the PAE measured by the two methods after different antibiotics is shown in Figure 2 for *S. aureus* and Figure 3 for *E. coli*. The correlation coefficients for the relationship between PAE_{vc} and PAE_{th} were $r^2=0.821$ ($p=0.0001$) for *S. aureus* and $r^2=0.939$ ($p<0.0001$) for *E. coli*. For *S. aureus* the regression line had an intercept of −0.03 and a slope of 0.76 ± 0.25 (95% confidence intervals [CI]) and for *E. coli* the regression line had an intercept of 0.53 and a slope of 1.07 ± 0.13 (95% CI) . The slopes were not statistically different from a slope of 1.0. If the two methods were equivalent in determining the PAE, the slope would be 1.0 with an intercept of 0.

However, certain differences between the two methods were evident. As shown in Figure 2, the [^{3}H] thymidine incorporation method showed a tendency to underestimate the PAE for *S. aureus* by 0.5 ± 0.5 h (mean ± 95% CI). In contrast, the PAE was overestimated in *E. coli* by this method (Figure 3) by 0.8 ± 1.1 h (mean ± 95% CI). The detection threshold of the [^{3}H]thymidine incorporation method was close to 10^5 CFU/mL for *S. aureus* and 10^6 CFU/mL for *E. coli*. For each $\log_{10}$ CFU/mL below these limits, 50–60 min were added to the value obtained by the standard method. A good correlation was demonstrated between the degree of over- or underestimation by the [^{3}H] thymidine incorporation method and $\log_{10}$ CFU/mL after drug removal ($r^2=0.684$ [$p<0.005$] and $r^2=0.876$ [$p<0.005$] for *S. aureus* and *E. coli*, respectively).

A comparison of the two methods for determination of CERT is shown in Figure 4. As shown, the two methods correlated well, with $r^2=0.867$ (slope = 0.936, intercept = −0.12) for *S. aureus* and $r^2=0.997$

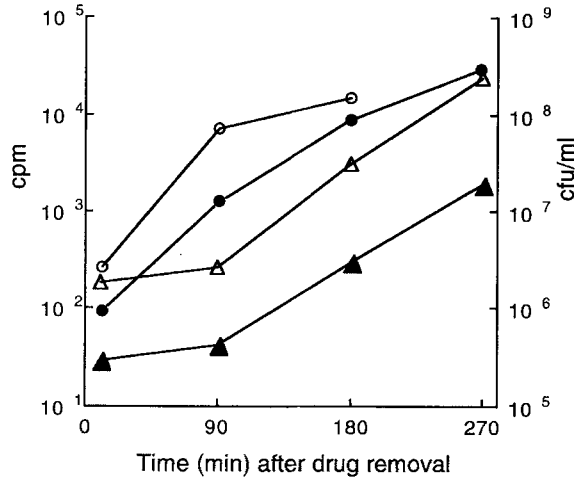

Figure 1 Examples of growth (CFU/mL) and [^{3}H] thymidine incorporation (counts/min) curves of *E. coli* ATCC 25922. Circles, unexposed control organisms; triangles, organisms during PAE after exposure to doxycyclin 4×MIC for 1 h. Closed symbols, viability counts ($\log_{10}$(CFU/mL)); open symbols, [^{3}H] thymidine incorporation (counts/min). In the example, the PAE determined by viable counts was 1.2 h and by [^{3}H] thymidine incorporation 1.6 h.

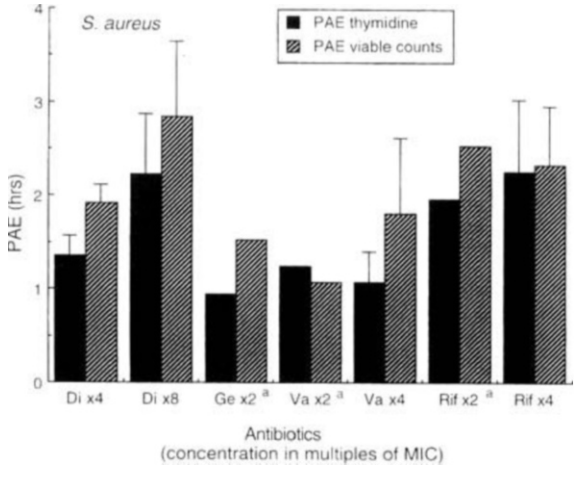

Figure 2 Comparison of the [^{3}H] thymidine incorporation method and viability counting for PAE determination in *S. aureus*. Bacterial counts post-drug removal were $>10^4$ CFU/mL (range: 2.5×10^4 to 6×10^5 CFU/mL). Columns represent means from two to three experiments. Bars represent range. Di, dicloxacillin; Ge, gentamicin; Va, vancomycin; Rif, rifampin. a: data from one experiment.

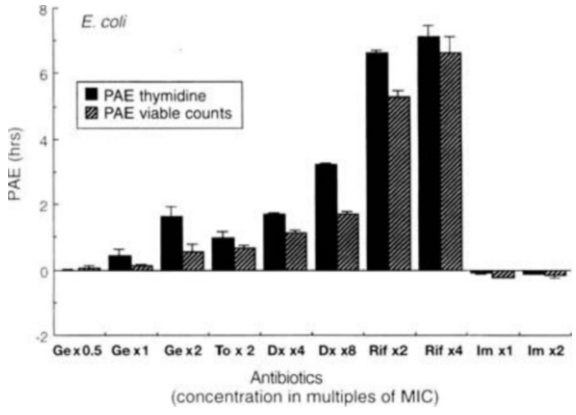

Figure 3 Comparison of the [^{3}H]thymidine incorporation method and viability counting for PAE determination in *E. coli*. Bacterial counts post-drug removal were $>10^4$ CFU/mL (range: 1.5×10^4 to 2.3×10^6 CFU/mL). Columns represent means from two to three experiments. Bars represent range. Ge, gentamicin; To, tobramycin; Dx, doxycycline; Rif, rifampin; Im, imipenem.

(slope = 0.979, intercept = −0.11) for *E. coli*. The duration of the CERT was similar irrespective of the method used: 2.6 ± 1.2 (mean ± SD by viable counts) and 2.3 ± 1.2 (mean ± SD by [^{3}H]thymidine) for *S. aureus*, and 3.4 ± 3.0 (mean ± SD by viable counts) and 3.2 ± 3.0 (mean ± SD by [^{3}H]thymidine) for *E. coli*.

DISCUSSION

Previously, we have demonstrated a good correlation between [^{3}H]thymidine incorporation and viable counts in a growing culture of *S. aureus* and *E. coli* [22]. In this study good correlation was demonstrated between the method of [^{3}H]thymidine incorporation for determination of the duration of the PAE and the 'gold-standard' of viable counts.

However, as compared to the PAEs quantitated by viable counts, the PAEs quantitated by [^{3}H]thymidine incorporation were usually underestimated for *S. aureus* (Figure 2) and overestimated for *E. coli* (Figure 3), but by less than 1 h; these inaccuracies are thus not likely to have serious consequences for screening.

On the other hand, a different discrepancy became evident regarding the rate of uptake of [^{3}H]thymidine between species, dependent on the bacterial count after drug removal. The detection limits of the method was 5 $\log_{10}$ CFU/mL for *S. aureus* and 6 $\log_{10}$ CFU/mL for *E. coli*. For each $\log_{10}$ CFU/mL below these numbers, the duration of the PAE was overestimated by 50–60 min as measured by the [^{3}H]thymidine incorporation method, probably representing the time needed for the organisms to reach sufficient biomass. The reasons for the different detection limits for *S. aureus* and *E. coli* are obscure, but may relate to differences in intracellular amounts of thymidine between species, variation in the rate of [^{3}H]thymidine uptake [22] or differences in biomass/CFU ratio between species. Furthermore, we have previously shown that patterns of bacterial DNA synthesis during PAE are different depending on the antibiotics, which might explain the differences in PAE determined by the two methods reported here [22].

A number of alternative methods have been employed for quantitation of the PAE [23]. Studies employing the bioluminescence assay of intracellular bacterial ATP have produced equivocal results: values both similar to and three to four times higher than the ones obtained by viable counts [6–9], depending on the organism–antibiotic combination. Moreover, the bactericidal action of antibiotics is frequently underestimated by this method, possibly due to the contribution of dead, still intact bacteria to the ATP level [6], leading to longer PAEs. An advantage of the [^{3}H]thymidine incorporation method is that unlysed dead bacteria do not incorporate [^{3}H]thymidine but may still contain intracellular ATP and contribute to optical density.

PAE determinations by optical density compared well with viable counts after exposure of *E. coli* to three antibiotics [11]. However, the threshold of detection for this method was high, approximating 6.7 $\log_{10}$ CFU/mL [12]. Using conductance for determination of PAE, the threshold of sensitivity was shown to be

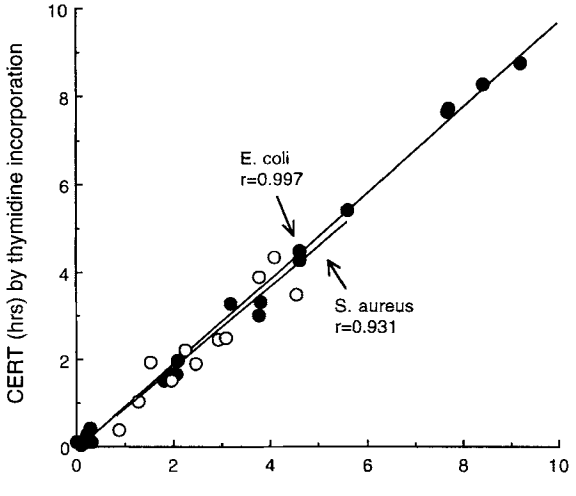

Figure 4 Correlation between CERT in *S. aureus* and *E. coli* as determined by viability counting and [^{3}H] thymidine incorporation. Closed circles: *E. coli*; open circles: *S. aureus*.

close to 10^5 CFU/mL [14], which is similar to the sensitivity of the [^{3}H]thymidine method.

CERT is a combined pharmacodynamic variable representing the sum of bacterial kill and PAE. Other studies have demonstrated good correlation between CERT measured by bioluminescence of ATP and viable counts, despite poor correlation of the two methods for PAE determination [19]. Our findings suggest that [^{3}H]thymidine incorporation may also be of use for measuring CERT.

In this study, a good concordance was observed between the PAEs and CERTs of selected antibiotics, whether measured by viable counts or by [^{3}H]thymidine incorporation. Thus, measuring these variables by [^{3}H]thymidine incorporation is an alternative, less laborious method than the conventional method of viable counts. If all facilities are at hand, the results can be obtained 2 h after the end of growth suppression. For determination of PAE, bacterial counts exceeding 10^4 CFU/mL are generally required after drug removal, significantly lower than for measurements of optical density and impedance. Pulsing the bacterial culture with [^{3}H]thymidine at 1.5-h intervals seems sufficient for PAE estimation. The results can thus be obtained by three to five pulsations for most antibiotics. Apart from these practical considerations, measuring the PAE of suitable organisms by [^{3}H]thymidine incorporation gives additional information on metabolic activity in the growth-suppressed bacteria not obtainable by the conventional method of viable counts [22].

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